Dual-Specificity Phosphatase 3 Deficiency or Inhibition Limits Platelet Activation and Arterial Thrombosis

Dual-Specificity Phosphatase 3 Deficiency or Inhibition Limits Platelet Activation and Arterial Thrombosis

Lucia Musumeci, PhD; Marijke J. Kuijpers, PhD; Karen Gilio, MD, PhD; Alexandre Hego, BSc; Emilie Théâtre, PhD; Lisbeth Maurissen, PhD; Maud Vandereyken, MSc; Catia V. Diogo, PhD; Christelle Lecut, PhD; William Guilmain, PhD; Ekaterina V. Bobkova, PhD; Johannes A. Eble, PhD; Russell Dahl, PhD; Pierre Drion, DVM, PhD; Justin Rascon, PhD; Yalda Mostofi, BSc; Hongbin Yuan, PhD; Eduard Sergienko, PhD; Thomas D.Y. Chung, PhD; Marc Thiry, PhD; Yotis Senis, PhD; Michel Moutschen, MD, PhD; Tomas Mustelin, MD, PhD; Patrizio Lancellotti, MD, PhD; Johan W.M. Heemskerk, PhD; Lutz Tautz, PhD*; Cécile Oury, PhD*; Souad Rahmouni, PhD*

Background—A limitation of current antiplatelet therapies is their inability to separate thrombotic events from bleeding occurrences. A better understanding of the molecular mechanisms leading to platelet activation is important for the development of improved therapies. Recently, protein tyrosine phosphatases have emerged as critical regulators of platelet function.

Methods and Results—This is the first report implicating the dual-specificity phosphatase 3 (DUSP3) in platelet signaling and thrombosis. This phosphatase is highly expressed in human and mouse platelets. Platelets from DUSP3-deficient mice displayed a selective impairment of aggregation and granule secretion mediated by the collagen receptor glycoprotein VI and the C-type lectin-like receptor 2. DUSP3-deficient mice were more resistant to collagen- and epinephrine-induced thromboembolism compared with wild-type mice and showed severely impaired thrombus formation on ferric chloride–induced carotid artery injury. Intriguingly, bleeding times were not altered in DUSP3-deficient mice. At the molecular level, DUSP3 deficiency impaired Syk tyrosine phosphorylation, subsequently reducing phosphorylation of phospholipase Cγ2 and calcium fluxes. To investigate DUSP3 function in human platelets, a novel small-molecule inhibitor of DUSP3 was developed. This compound specifically inhibited collagen- and C-type lectin-like receptor 2–induced human platelet aggregation, thereby phenocopying the effect of DUSP3 deficiency in murine cells.

Conclusions—DUSP3 plays a selective and essential role in collagen- and C-type lectin-like receptor 2–mediated platelet activation and thrombus formation in vivo. Inhibition of DUSP3 may prove therapeutic for arterial thrombosis. This is the first time a protein tyrosine phosphatase, implicated in platelet signaling, has been targeted with a small-molecule drug. **(*Circulation*. 2015;131:656-668. DOI: 10.1161/CIRCULATIONAHA.114.010186.)**

Key Words: antagonists and inhibitors ■ blood platelets ■ signal transduction ■ thrombosis

Received March 22, 2014; accepted December 12, 2014.

From the Immunology and Infectious Diseases Unit, GIGA-Signal Transduction (L. Musumeci, L. Maurissen, M.V., C.V.D., M.M., S.R.), Laboratory of Thrombosis and Haemostasis, GIGA-Cardiovascular Sciences (A.H., L. Maurissen, C.V.D., C.L., W.G., C.O.), Unit of Animal Genomics, GIGA-Genetics and Faculty of Veterinary Medicine (E.T.), Unit of Hepato-Gastroenterology, CHU de Liège and Faculty of Medicine (E.T.), GIGA-Animal Facility (B23) (P.D.), Laboratory of Cell and Tissue Biology, GIGA-Neurosciences (M.T.), and Department of Cardiology, Heart Valve Clinic, CHU Sart Tilman, GIGA Cardiovascular Sciences (P.L.), University of Liège, Liège, Belgium; Laboratory of Cellular Thrombosis and Haemostasis, Cardiovascular Research Institute Maastricht CARIM, Maastricht University, Maastricht, the Netherlands (M.J.K., K.G., L. Maurissen, J.W.M.H.); Conrad Prebys Center for Chemical Genomics (E.V.B., R.D., J.R., Y.M., H.Y., E.S., T.D.Y.C.) and NCI-Designated Cancer Center (L.T.), Sanford-Burnham Medical Research Institute, La Jolla, CA; Institute for Physiological Chemistry and Pathobiochemistry, University of Münster, Münster, Germany (J.A.E.); and Centre for Cardiovascular Sciences, Institute of Biomedical Research, School of Clinical and Experimental Medicine, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham, UK (Y.S.).

*Drs Tautz, Oury, and Rahmouni contributed equally.

The online-only Data Supplement is available with this article at http://circ.ahajournals.org/lookup/suppl/doi:10.1161/CIRCULATIONAHA.114.010186/-/DC1.

Correspondence to Souad Rahmouni, PhD, University of Liège, Immunology and Infectious Diseases Research Unit, GIGA B34, Avenue de l'Hôpital, 1, B-4000 Liège, Belgium. E-mail srahmouni@ulg.ac.be or Cécile Oury, PhD, University of Liège, Laboratory of Thrombosis and Hemostasis, GIGA B34, Avenue de l'Hôpital, 1, B-4000 Liège, Belgium. E-mail cecile.oury@ulg.ac.be or Lutz Tautz, PhD, Sanford-Burnham Medical Research Institute, NCI-Designated Cancer Center, 10901 N Torrey Pines Rd, La Jolla, CA 92037. E-mail tautz@sanfordburnham.org

Antiplatelet therapy has been effective in reducing the mortality and morbidity of acute myocardial infarction, the most common cause of death in developed countries.[1] However, US Food and Drug Administration–approved antiplatelet agents have serious side effects, including gastrointestinal toxicity, neutropenia, thrombocytopenia, and common bleeding.[1] There also remains a considerable incidence of arterial thrombosis in patients receiving currently available antiplatelet therapy.[1] A better understanding of the molecular mechanisms leading to platelet activation is essential for the development of new therapeutics.

Clinical Perspective on p 668

Platelet activation depends on rapid phosphorylation and dephosphorylation of key signaling proteins, in particular tyrosine.[2] Although the repertoire of protein tyrosine kinases has been well described in platelet activation, the expression, regulation, specificity, and function of platelet-expressed protein tyrosine phosphatases (PTPs) are largely unknown. A recent proteomic analysis found that 14 of 37 classic phosphotyrosine-specific PTPs are expressed in human platelets.[3] Expression and function of the dual-specificity phosphatases,[4,5] the largest subgroup of the PTP superfamily, are unexplored.

Dual-specificity phosphatase 3 (DUSP3), also known as *Vaccinia* H1–related phosphatase, is a dual-specificity phosphatase encoded by the *DUSP3/Dusp3* gene. DUSP3 (185 amino acids; Mr, 21 kDa), which contains only a catalytic (PTP) domain,[6] has been reported to dephosphorylate the mitogen-activated protein kinases extracellular signal-regulated kinase 1/2 (ERK1/2) and Jun N-terminal kinase 1/2 (JNK1/2).[7] Additional reported substrates include epidermal growth factor receptor and ErbB2.[8] DUSP3 is implicated in cell cycle regulation, and its expression is altered in human cancer.[9–11] However, because all of these studies were performed either in vitro with recombinant proteins or in cell lines with transient overexpression or siRNA knockdown, the true physiological function of DUSP3 has remained elusive. We recently generated a full *Dusp3*-knockout (*Dusp*3-KO) mouse.[12] *Dusp*3-KO mice were healthy and fertile and showed no spontaneous phenotypic abnormality. However, DUSP3 deficiency prevented neoangiogenesis and basic fibroblast growth factor–induced microvessel outgrowth.[12] In the present study, we identified DUSP3 as a key and nonredundant player in glycoprotein (GP) VI– and C-type lectin-like receptor 2 (CLEC-2)–mediated signaling pathways in mouse and human platelets. We show that DUSP3 deficiency limits platelet activation and arterial thrombosis. Moreover, we developed a specific small-molecule inhibitor of DUSP3 that was able to phenocopy DUSP3 deficiency in platelets.

Methods

Platelet RNA Sampling and Microarray

Platelets from 256 healthy volunteers were isolated from citrate-anticoagulated blood. Donors were informed about the objectives of the study and signed an informed consent. The study was approved by the ethics committee review board of the Liège University Hospital. RNA extraction and microarray procedures are described in the online-only Data Supplement.

Mice

C57BL/6-*Dusp3*-KO mice were generated by homologous recombination.[12] Heterozygous mice were mated to generate +/+ and –/– littermates used for experimentation (male mice 8–12 weeks old). All experiments were approved by the local ethics committee.

Isolation of Human and Mouse Platelets

Human platelets were prepared from peripheral blood freshly drawn from healthy donors, as previously described.[13] Mouse washed platelets were prepared as previously described.[14]

Isolation of Human and Murine B and T Cells

Human B and T cells were sorted from freshly collected blood with the use of EasySep B and T cell–negative selection kits (Stemcell Technologies). Mouse B and T cells were sorted from spleens.

Platelet Aggregation Analyses

Light transmission was recorded during platelet aggregation induced by collagen, convulxin, collagen-related peptide (CRP), rhodocytin, thrombin, U46619, or ADP in the presence of 2 mmol/L $CaCl_2$ on a Lumi-Aggregometer (Chrono-log).

Flow Cytometry

Washed platelets were stimulated for 15 minutes with different concentrations of collagen, CRP, thrombin, or ADP under nonstirring conditions. Saturating concentrations of FITC-conjugated P-selectin and phycoerythrin-conjugated JON/A antibodies were added. Samples were analyzed on a FACSCantoII flow cytometer (BD Biosciences).

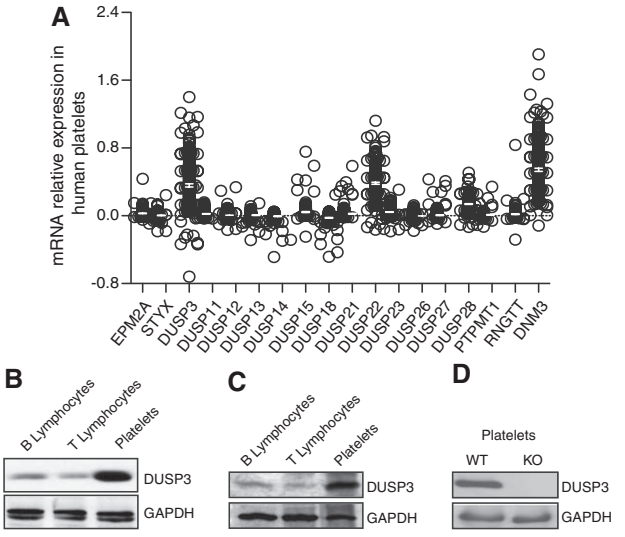

Figure 1. Dual-specificity phosphatase 3 (DUSP3) expression in human and mouse platelets. **A**, Microarray data of mRNA expression of 17 atypical dual-specificity phosphatases (DSPs) in human platelets isolated from 256 healthy volunteers. Each open circle represents 1 individual. DNM3 was used as positive control for platelet-expressed mRNA. Data are presented as ratio of the fluorescence intensity for the DSP probe of interest and the mean fluorescence intensity for the housekeeping genes of each sample. A negative value corresponds to an expression below background level. Mean±SEM values are shown. **B** through **D**, DUSP3 protein expression in human B and T lymphocytes and in platelets isolated from peripheral blood (**B**), in mouse splenic B and T cells and in washed platelets (**C**), and in wild-type (WT) and *Dusp3*-knockout (KO) mouse washed platelets (**D**). Western blot analysis was performed with anti-human (**B**) and anti-mouse DUSP3 (**C** and **D**). GAPDH was used as loading control. Representative blots of 3 independent experiments are shown.

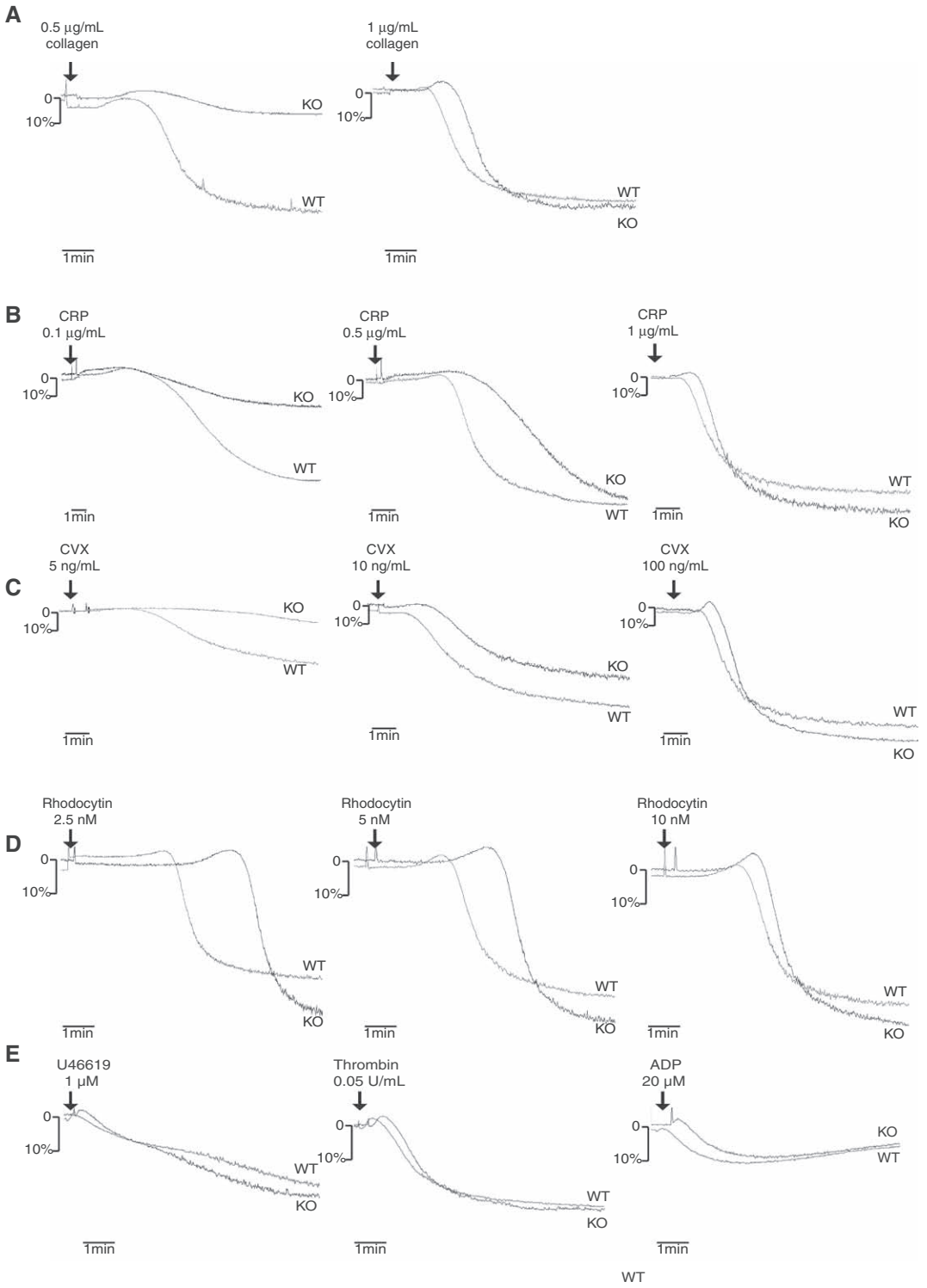

Figure 2. Dual-specificity phosphatase 3 (DUSP3)–deficient platelets exhibit impaired glycoprotein (GP) VI– and C-type lectin-like receptor 2 (CLEC-2)–mediated platelet aggregation. **A** through **E**, Washed platelets prepared from wild-type (WT) or *Dusp3*-knockout (KO) mice were stimulated with collagen (0.5 and 1 µg/mL; **A**), collagen-related peptide (CRP; 0.1, 0.3, and 1 µg/mL; **B**), convulxin (CVX; 5, 10, and 100 ng/mL; **C**), rhodocytin (2.5, 5, and 10 nmol/L; **D**), or the thromboxane A_2 analog U46619 (1 µmol/L), thrombin (0.05 U/mL), or ADP (20 µmol/L; **E**). Representative platelet aggregation curves of 3 individual experiments are shown.

Electron Microscopy

Platelet pellets were fixed for 60 minutes in 2.5% glutaraldehyde in Sörensen buffer (0.1 mol/L, pH 7.4), postfixed for 30 minutes with 1% osmium tetroxide, dehydrated in a series of ethanol concentrations, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined on a Jeol-CX100II transmission electron microscope (60 kV).

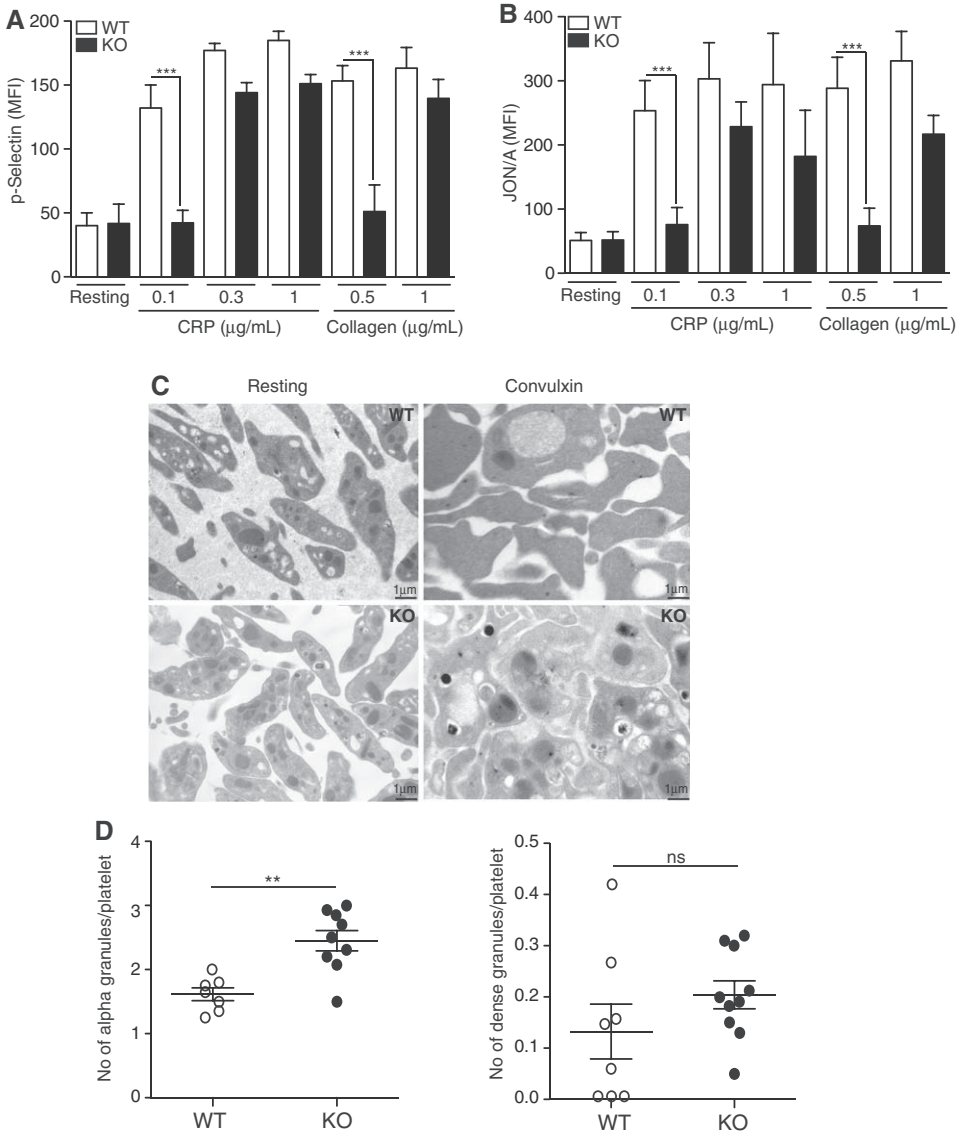

Figure 3. Impaired glycoprotein (GP) VI–mediated platelet activation in dual-specificity phosphatase 3 (DUSP3)–deficient platelets. **A** and **B**, Washed platelets from wild-type (WT) or *Dusp3*-knockout (KO) mice were stimulated with 0.1, 0.3, or 1 μg/mL collagen-related peptide (CRP) or 0.5 or 1 μg/mL collagen under nonstirring conditions or left untreated. Surface expression of P-selectin and active integrin $\alpha_{IIb}\beta_3$ (JON/A) was quantified by flow cytometry. Mean fluorescence intensity (MFI) histograms for p-selectin (**A**) and JON/A (**B**) are shown. Data were analyzed with ANOVA and the Bonferroni multiple-comparison test and represent the mean±SEM of 4 independent experiments performed on platelet pools from 3 mice each; **P*<0.05, ***P*<0.01. **C**, Electron microscopy analysis of WT and *Dusp3*-KO washed platelets. Platelet ultrastructure was visualized in the resting state or on convulxin stimulation (100 ng/mL). **D**, Scatterplots of α- and dense granules counted on 5 separate micrographs. Data were analyzed with the unpaired Student *t* test and represent the mean±SEM of 3 independent experiments performed on platelet pools from 3 mice; **P*<0.05, ***P*<0.01.

Whole-Blood Platelet Aggregate Formation Under Flow

Thrombus formation under flow conditions was assessed with anticoagulated mouse blood (4 U/mL heparin, 20 μmol/L PPACK), as previously described.[15] Area coverage from phase-contrast images was analyzed with ImagePro (Media Cybernetics).[16] Area coverage by platelets stained with OG488-annexin A5 was determined with Quanticell (Visitech).

Ca^{2+} Flux

Apyrase (0.5 U/mL)-treated murine washed platelets were loaded with 3.5 μmol/L fura-2-acetoxymethyl ester in the presence of Pluronic F-127 for 15 minutes. Fluorescence was recorded on an Aminco spectrofluorimeter (SLM Instruments), as described elsewhere.[17]

Arterial Thrombosis Models

Pulmonary embolism was induced by injection of a mixture of collagen (170 μg/kg) and epinephrine (60 μg/kg) into the plexus retroorbital veins of anesthetized mice (ketamine, 60 mg/kg; xylazine, 5 mg/kg). Time to death was monitored. Lungs were perfused with 4% formaldehyde solution and collected for histological studies.

Injury of carotid arteries of anesthetized mice was performed by applying a filter paper soaked in 10% ferric chloride ($FeCl_3$) solution to the exposed artery for 5 minutes.[18] Fluorescence of exogenously carboxyfluorescein succinimidyl ester–labeled platelets was monitored with a BX61WI microscope (Olympus). Digital images were captured with a Hamamatsu 9100-13 electron-multiplying charge-coupled device camera using a Lambda DG-4 (Sutter instrument) light source and Slidebook software 5.5 (3i).

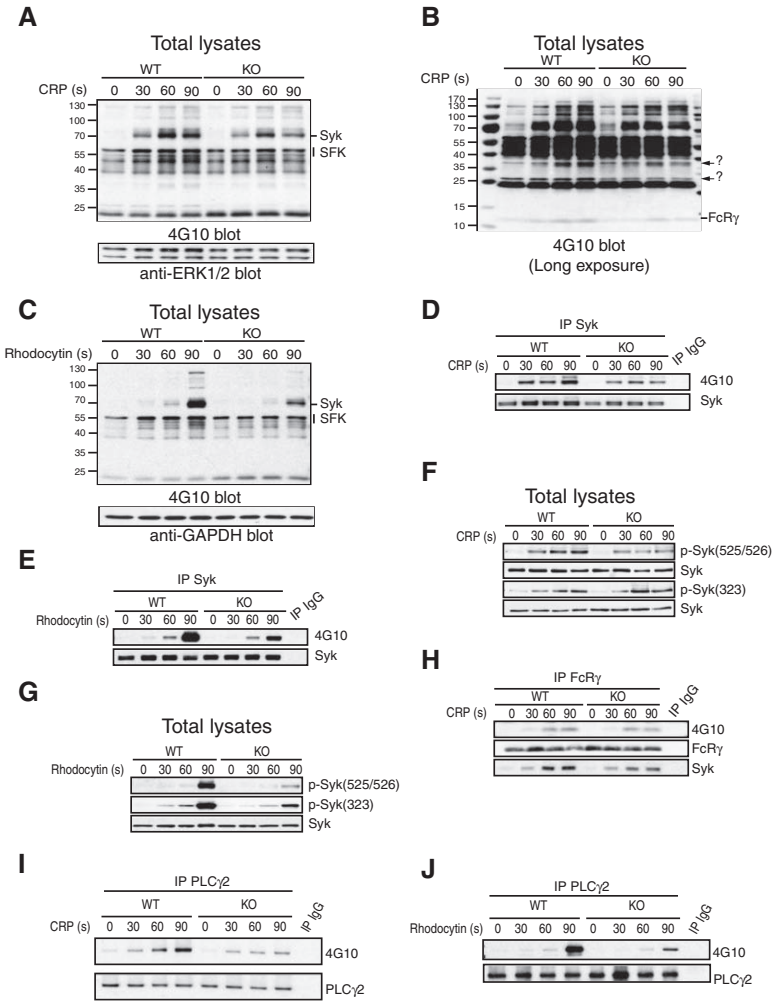

Figure 4. Dual-specificity phosphatase 3 (DUSP3) deficiency impairs Syk tyrosine phosphorylation. Total lysates (TLs) were prepared from wild-type (WT) or *Dusp3*-knockout (KO) mice platelets. Cells were nonactivated or activated by collagen-related peptide (CRP; 0.3 μg/mL) or rhodocytin (10 nmol/L) for 30, 60, or 90 seconds. **A** through **C**, Western blot analysis with anti-phosphotyrosine (pY) antibody (4G10) and with extracellular signal-regulated kinase 1/2 (ERK1/2; **A**) or GAPDH (**C**) as loading control. Arrows in **B** indicate unknown protein bands with attenuated pY levels in DUSP3-deficient platelets. **D** and **E**, Representative pY blot of Syk immunoprecipitates (IPs) from TLs of CRP-activated (**D**) or rhodocytin-activated (**E**) platelets. **F** and **G**, Representative blot of Syk phosphorylation on Tyr-323 and Tyr-525/526 in CRP-activated (**F**) or rhodocytin-activated (**G**) platelets. Normalization was performed with total Syk. **H**, pY and Syk Western blots on Fc receptor γ-chain (FcRγ) IPs. **I** and **J**, pY blot of phospholipase Cγ2 (PLCγ2) IPs from TLs of CRP-activated (**I**) or rhodocytin-activated (**J**) platelets. Results shown are representative of 3 independent experiments performed on platelet pools from 3 mice each.

Mouse Irradiation and Bone Marrow Transplantation

Donor mice (7–8 weeks old) were euthanized by cervical dislocation. Tibia and fibula were collected, and bone marrow was flushed with PBS. Then, 10×10^6 single bone marrow cells were transplanted to 4- to 5-week-old lethally irradiated (866.3 cGy) recipient mice. Chimeric mice were used in the $FeCl_3$ model 3 to 4 weeks after transplantation. Chimerism was evaluated by Western blot of DUSP3 in lysates of peritoneal cavity cells.

Tail Bleeding

Mice were anesthetized with isoflurane. A 3-mm portion of the tail tip was excised and submerged in a 37°C water bath. Bleeding was monitored for 15 minutes.

Platelet Activation, Cell Lysis, Immunoprecipitation, and Western Blotting

Mouse washed platelets were activated with CRP or rhodocytin in Tyrode buffer for 30, 60, or 90 seconds under 400-rpm stirring conditions at 37°C. Western blotting and immunoprecipitations were performed according to standard procedures.[19]

Statistical Analysis

Data are presented as mean±SEM of at least 3 independent experiments. Data were analyzed by use of the unpaired Student *t* test or ANOVA and the Bonferroni multiple-comparison test, as indicated in each figure legend. Differences in survival were determined with Kaplan-Meier analysis (log-rank Mantel test). Values of $P<0.05$ were considered significant. Calculations were performed with GraphPad-Prism (GraphPad Software, Inc).

Results

DUSP3 Expression in Platelets

Transcriptomic analysis of platelets from 256 healthy human individuals revealed that DUSP3-encoding mRNA is highly expressed in platelets (Figure 1A). An abundance of DUSP3 in human and mouse platelets was confirmed by Western blot analysis (Figure 1B and 1C). Expression levels of DUSP3 were substantially higher in platelets compared with B and T lymphocytes (Figure 1B and 1C); DUSP3 function in B and T lymphocytes has previously been described.[7] Thus, we set out to investigate the role of DUSP3 in platelets using both genetic deletion in mice and pharmacological inhibition of DUSP3 in isolated human platelets.

Activation and Aggregation of DUSP3-Deficient Mouse Platelets

Using our previously generated *Dusp3*-KO mice,[12] we confirmed that platelets isolated from these animals do

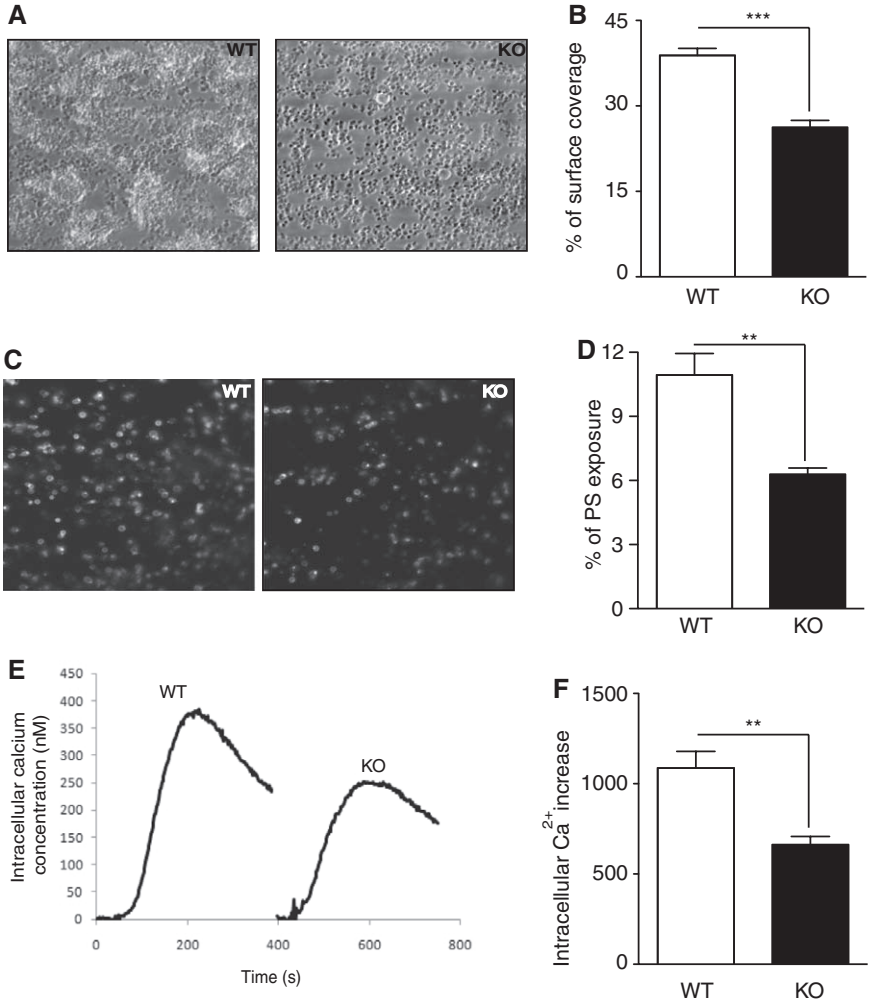

Figure 5. Impaired platelet aggregate formation on whole blood from dual-specificity phosphatase 3 (DUSP3)–deficient mice. **A** through **D**, Anticoagulated blood from wild-type (WT) or *Dusp3*-knockout (KO) mice was perfused over a collagen-coated coverslip through a parallel-plate transparent flow chamber at a wall shear rate of 1000 seconds^{-1} for 4 minutes. Representative phase-contrast images of fixed platelets (**A**) and percentages of surface coverage by platelets (**B**) are shown. Exposure of phosphatidylserine (PS) was detected by postperfusion with heparin and OG488-labeled Annexin-V–containing rinsing buffer. Representative fluorescent images (**C**) and percentage of area coverage by labeled platelets (**D**) are shown. **E** and **F**, Intracellular Ca^{2+} increase in WT and *Dusp*3-KO platelets on convulxin stimulation (50 ng/mL). Representative curves (**E**) and histogram depicting the area under the curve (**F**) are shown. WT values are arbitrarily set to 100%. An unpaired Student *t* test was used for comparison. Results are representative of 5 independent experiments performed on platelet pools from 3 mice each. Data represent mean±SEM of 3 independent experiments; ***P*<0.01, ****P*<0.001.

not express DUSP3 (Figure 1D). Hematologic parameters were normal except for slight but significant differences in monocytes ($P<0.05$) and mean platelet volume ($P<0.0001$; Table I in the online-only Data Supplement). *Dusp3*-KO mice did not show any spontaneous bleeding or thrombotic disorders. However, in platelet aggregation assays, DUSP3-deficient platelets failed to aggregate in response to low concentrations of collagen (0.5 µg/mL) and selective GPVI agonists, including convulxin (5 ng/mL) and CRP (0.1 µg/mL; Figure 2A–2C). Additionally, *Dusp3*-KO platelets exhibited delayed aggregation induced by low concentrations of rhodocytin (2.5 and 5 nmol/L), a selective CLEC-2 receptor agonist (Figure 2D). GPVI and CLEC-2 surface expression on *Dusp3*-KO platelets was similar to that of wild-type (WT) platelets (Figures IA and IIA in the online-only Data Supplement). In contrast, aggregation induced by ADP (5–50 µmol/L), the thromboxane A_2 mimetic U46619 (0.75–2 µmol/L), or thrombin (0.01–0.1 U/mL) occurred normally (Figure 2E and data not shown), indicating normal G-protein–coupled receptor–mediated responses.

To investigate the mechanism responsible for the impairment of collagen- and CRP-induced aggregation of DUSP3-deficient platelets, we analyzed their ability to release granule content by measuring P-selectin surface expression, and we examined their capacity to activate integrin $\alpha_{IIb}\beta_3$ by using the JON/A antibody, which is specific for the high-affinity conformation of mouse $\alpha_{IIb}\beta_3$. In DUSP3-deficient compared with WT platelets, P-selectin expression was reduced after stimulation with a low concentration of collagen (0.5 µg/mL) or various concentrations of CRP (0.1, 0.3, and 1 µg/mL; Figure 3A). Integrin $\alpha_{IIb}\beta_3$ activation

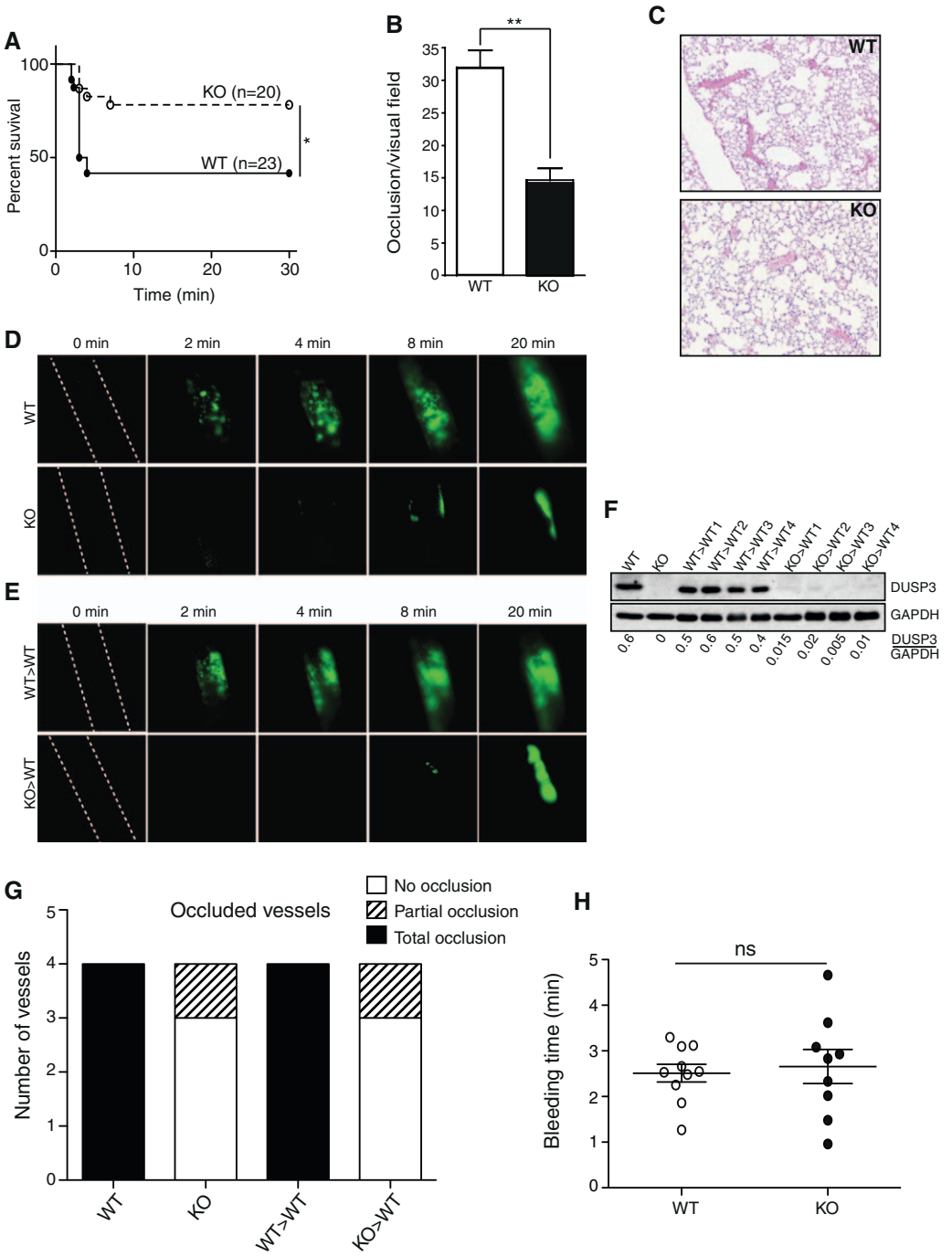

Figure 6. Impaired arterial thrombosis and preserved hemostasis in dual-specificity phosphatase 3 (DUSP3)–deficient mice. **A** through **C**, Pulmonary thromboembolism induced by injection of a mixture of collagen and epinephrine. Mortality incidence rates compared by use of Kaplan-Meir analysis with a log-rank test (n=20 for *Dusp3*-knockout [KO] and n=23 for wild-type [WT] mice; **A**), quantification of the number of occluded vessels per visual field on lung sections from WT and *Dusp*3-KO mice (**B**), and representative field on lung sections from WT and *Dusp3*-KO mice (**C**) are shown. Data (from 3 to 6 lung sections from 3 mice from each group) were analyzed by use of an unpaired Student *t* test; $^{**}P<0.01$. **D** and **E**, $FeCl_3$ injury of carotid arteries. Representative snapshot images in WT and *Dusp3*-KO mice (**D**) and in WT mice transplanted with WT (WT>WT) or with *Dusp3*-KO (KO>WT) bone marrow (BM) cells (**E**) are shown. **F**, Western blot analysis of DUSP3 expression in peritoneal total lysates from WT>WT and KO>WT BM-transplanted mice used in the $FeCl_3$ assay. Normalization was performed with GAPDH. **G**, Numbers of intact and partially occluded vessels are shown for 4 mice from each group. **H**, Tail bleeding time of WT (●) and *Dusp3*-KO (○) mice. Each dot or circle represents 1 mouse. Results are presented as mean±SEM.

was reduced with low concentrations of collagen (0.5 µg/mL) and CRP (0.1 µg/mL; Figure 3B). Electron microscopy analysis of resting DUSP3-deficient platelets revealed normal ultrastructure but a slightly increased number of α-granules (Figure 3C and 3D). When activated with convulxin, degranulation remained incomplete among the few

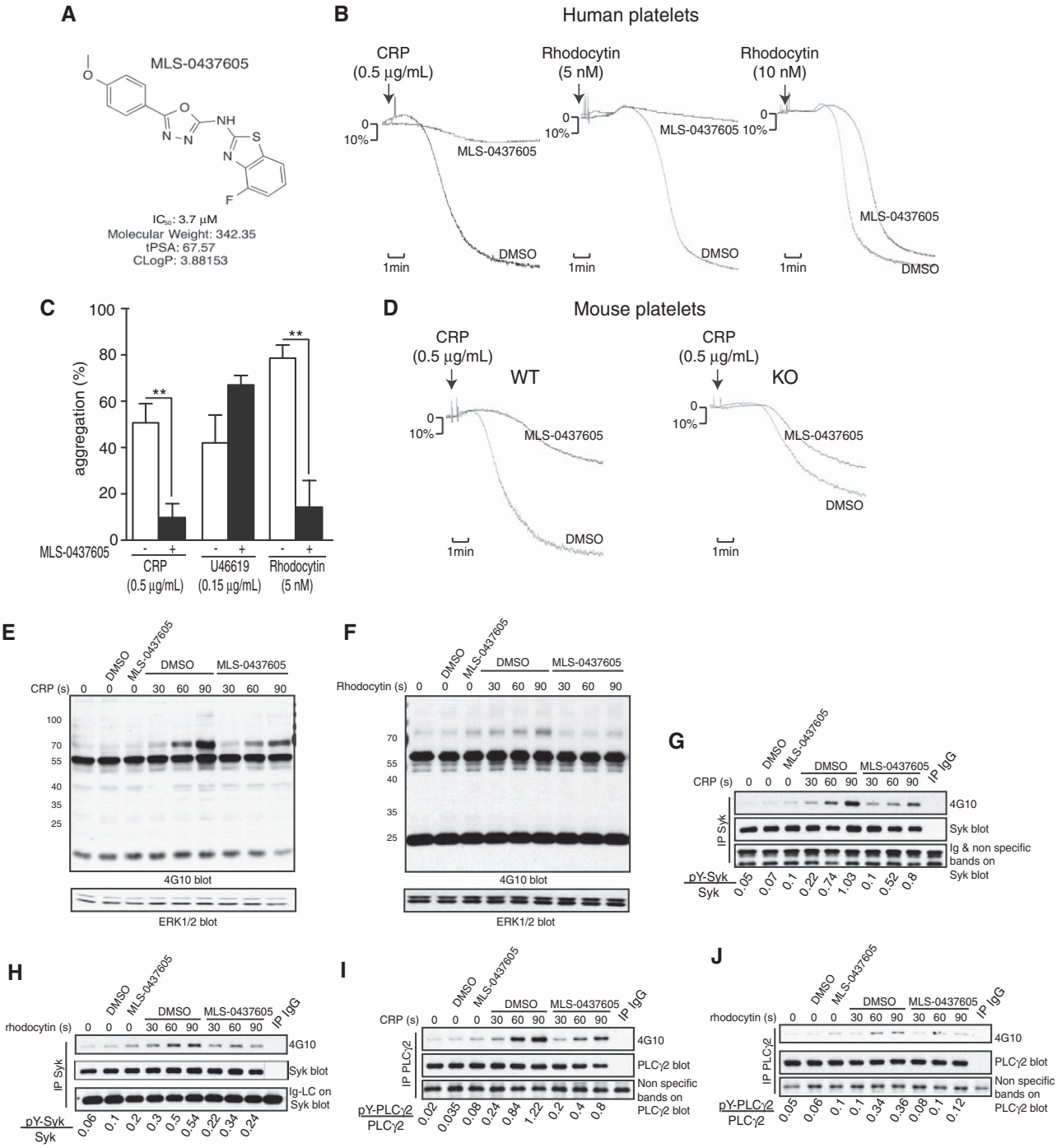

Figure 7. The dual-specificity phosphatase 3 (DUSP3)–specific inhibitor MLS-0437605 inhibits platelet activation in response to collagen-related peptide (CRP) and rhodocytin. **A**, Chemical structure and key properties of MLS-0437605. **B** through **D**, Washed human platelets (**B** and **C**) or wild-type (WT) and *Dusp3*-knockout (KO) mouse washed platelets (**D**) were preincubated for 30 minutes with dimethyl sulfoxide (DMSO; vehicle) or with MLS-0437605 (3.7 μmol/L) before being stimulated with CRP (0.5 μg/mL), rhodocytin (5 or 10 nmol/L), or U46619 (0.15 μg/mL). Representative platelet aggregation curves (**B** and **D**) and quantification of platelet aggregation from 3 healthy human donors (**C**) are shown. Results were analyzed by use of 1-way ANOVA and the Bonferroni multiple-comparison test and are presented as mean±SEM; $^{**}P<0.01$. **E** through **J**, Total lysates (TLs) were prepared from vehicle- or MLS-0437605–pretreated human platelets. Cells were nonactivated or activated with CRP or rhodocytin for the indicated times. Western blot analysis was performed with 4G10 antibody for global phosphotyrosine of CRP (**E**) and rhodocytin (**F**) activated platelets. Extracellular signal-regulated kinase 1/2 (ERK1/2) was used as a loading control. **G** and **H**, Representative phosphotyrosine (pY) blot of Syk immunoprecipitates (IPs) from TLs of CRP-activated (**G**) or rhodocytin-activated (**H**) platelets. **I** and **J**, pY blot of phospholipase Cγ2 (PLCγ2) IPs from TLs of CRP-activated (**I**) or rhodocytin-activated (**J**) platelets. Data are representative of 2 individual healthy donors.

DUSP3-deficient platelet aggregates compared with WT (Figure 3C). These findings indicate that DUSP3 deficiency impairs GPVI- and CLEC-2–dependent mouse platelet activation and aggregation.

GPVI and CLEC-2 Signaling in DUSP3-Deficient Platelets

Earlier studies suggested that DUSP3 dephosphorylates ERK1/2 and JNK1/2 but not p38.[7] Therefore, we evaluated

the activation of these mitogen-activated protein kinases using phospho-specific antibodies at basal levels and after CRP stimulation. No differences in mitogen-activated protein kinase activation between DUSP3-deficient and WT platelets were found (Figure III in the online-only Data Supplement). We then analyzed global tyrosine phosphorylation and found decreased phosphorylation of a ≈70-kDa band in DUSP3-deficient compared with WT platelets after CRP or rhodocytin stimulation (Figure 4A and 4C). Longer exposure of the phosphotyrosine blot revealed additional bands (at ≈12, ≈26, and ≈40 kDa) with decreased phosphorylation in DUSP3-deficient compared with WT platelets after CRP stimulation (Figure 4B). We then tested whether the observed change in phosphotyrosine of the ≈70-kDa band may correspond to phosphotyrosine levels in the tyrosine kinase Syk (Mr, 72.1 kDa), a key signaling molecule in GPVI- and CLEC-2–mediated platelet activation. Indeed, phosphotyrosine of immunoprecipitated Syk was significantly reduced in DUSP3-deficient compared with WT platelets after GPVI and CLEC-2 stimulation ($P<0.05$; Figure 4D and 4E and Figure IVA and IVB in the online-only Data Supplement). Probing total lysates of DUSP3-deficient or WT platelets with phospho-Syk–specific antibodies revealed that, after activation with CRP, Syk phosphorylation was reduced on the activatory residues Tyr-525/526, whereas phosphorylation of the negative regulatory Tyr-323 was not affected (Figure 4F and Figure IVF and IVG in the online-only Data Supplement). In rhodocytin-stimulated platelets, Syk phosphorylation was reduced on both Tyr-525/526 and Tyr-323 in the absence of DUSP3 (Figure 4G and Figure VH and VI in the online-only Data Supplement).

Syk is recruited to the GPVI/Fc receptor γ-chain (FcRγ) complex via phosphorylation of FcRγ-associated immunoreceptor tyrosine-based activation motifs by Src-family kinases (SFKs) and is then activated via autophosphorylation.[20,21] We found that phosphorylation of FcRγ-associated immunoreceptor tyrosine-based activation motifs was reduced in DUSP3-deficient compared with WT platelets in response to CRP (Figure 4H and Figure IVJ in the online-only Data Supplement). In agreement with this observation, recruitment of Syk to FcRγ was impaired in DUSP3-deficient platelets (Figure 4H and Figure IVK in the online-only Data Supplement). Additionally, inducible tyrosine phosphorylation in phospholipase Cγ2, a key signaling molecule downstream of Syk, was reduced in both CRP- and rhodocytin-stimulated DUSP3-deficient compared with WT platelets (Figures 4I and 4J and Figure IVL and IVM in the online-only Data Supplement). In contrast, activation of SFKs, including Lyn, Fyn, and Src, was not altered (Figure VA and VB in the online-only Data Supplement), indicating that the reduced activation and recruitment of Syk in DUSP3-deficient platelets was not attributable to aberrant activation of SFKs.

Collagen-Induced Aggregation Under Flow, Calcium Fluxes, and Phosphatidylserine Exposure in DUSP3-Deficient Platelets

To further assess the role of DUSP3 in GPVI-dependent platelet responses, platelet aggregate formation and exposure of procoagulant phosphatidylserine on a collagen surface were analyzed in whole mouse blood under flow. The area covered by platelets was reduced by ≈40% for blood from *Dusp3*-KO compared with WT mice (Figure 5A and 5B), which was in agreement with reduced GPVI activation in DUSP3-deficient platelets.[22] Accordingly, overall phosphatidylserine exposure on adhered platelets was also diminished (Figure 5C and 5D). Because phosphatidylserine exposure requires Ca^{2+} influx,[23] we investigated whether Ca^{2+} flux was affected by DUSP3 deficiency. Convulxin-induced Ca^{2+} flux was greatly reduced (50%) in DUSP3-deficient compared with WT platelets (Figure 5E and 5F). Thapsigargin-induced Ca^{2+} increase occurred normally in DUSP3-deficient platelets (Figure VIA in the online-only Data Supplement), suggesting intact intrinsic Orai 1–mediated store-operated Ca^{2+} entry. Additionally, there were no differences in Ca^{2+} increases induced by thrombin, ADP, or U46619 between WT and *Dusp3*-KO platelets (Figure VIB–VID in the online-only Data Supplement). These data further support a positive role of DUSP3 in GPVI-mediated platelet activation under physiological flow conditions.

DUSP3 Deficiency and Thrombus Formation In Vivo

To evaluate the importance of DUSP3 in platelet function in vivo, we used a model of pulmonary thromboembolism induced by intravenous injection of a mixture of collagen and epinephrine. About 80% of DUSP3-deficient compared with 45% of WT mice survived (Figure 6A). Analyses of lung sections revealed significantly decreased numbers of occluded microvessels in DUSP3-deficient compared with WT mice ($P<0.001$; Figure 6B and 6C). We then examined thrombus formation in real time by intravital microscopy in a model of $FeCl_3$-induced injury of the carotid arteries. In this model, collagen is exposed to circulating blood, and thrombus formation depends highly on GPVI. In DUSP3-deficient mice, blood vessels were never occluded as a result of failure to form stable thrombi, whereas full occlusion occurred at 8 to 10 minutes after $FeCl_3$ application in WT vessels (Figure 6D and 6G). To test whether the defect in thrombus formation was due specifically to impaired platelet function, we generated chimeric mice by transferring *Dusp3*-KO bone marrow (KO>WT) or WT bone marrow (WT>WT) to lethally irradiated WT mice. Successful transplantation was evaluated by quantification of DUSP3 expression in peritoneal cell lysates from KO>WT and WT>WT mice (Figure 6F). Similar to *Dusp3*-KO, we found that thrombus formation was severely impaired in blood vessels of KO>WT mice (Figure 6E and 6G), confirming that the thrombosis defect in DUSP3-deficient animals was attributable to platelet dysfunction. Importantly, tail bleeding time, a measure of primary hemostasis in vivo, was identical for WT and DUSP3-deficient mice (Figure 6H).

Pharmacological Inhibition of DUSP3

To corroborate DUSP3 function in human platelets, we investigated the possibility of specifically inhibiting DUSP3 activity with small molecules. To identify DUSP3 inhibitors,

we used high-throughput screening, with a colorimetric phosphatase assay with *p*-nitrophenolphosphate as substrate, and screened 291 018 drug-like molecules.[24] Of the 1524 primary high-throughput screening hits (≥50% inhibition), 1048 compounds were available from BioFocus DPI and ordered for confirmatory assays. The hits were tested in 2 reconfirmation single-dose screens in triplicate with the use of both the primary colorimetric assay and an orthogonal fluorescent assay with 3-O-methylfluorescein phosphate as substrate. Compounds with an average of ≥50% inhibition of DUSP3 activity were further tested in a 10-point dose-response assay in both colorimetric and fluorescent formats. IC_{50} values were determined, and 67 "cross-active" compounds were identified with IC_{50} values <20 μmol/L in both assays. On visual inspection of each molecule, 32 compounds were discarded from further consideration because of their known promiscuous PTP inhibitory activity. The remaining 35 compounds were taken into selectivity profiling studies for further prioritization. Compound selectivity for inhibiting DUSP3 over the related DUSP6 and 3 additional PTPs, HePTP, LYP, and STEP, was evaluated (Table II in the online-only Data Supplement).

On the basis of the selectivity and potency of compounds, 2 scaffolds were selected for structure-activity relationship studies: MLS-0103602 and MLS-0049585 (Table II in the online-only Data Supplement). MLS-0103602 (IC_{50}=0.37 μmol/L) was the most potent inhibitor with some degree of selectivity for DUSP3; MLS-0049585 (IC_{50}=2.68 μmol/L) exhibited the best selectivity for DUSP3. On the basis of the benzothioamide structure of MLS-0103602, 37 analogs were tested and counterscreened. All analogs were at least an order of magnitude less potent than the original hit, with no improvement in selectivity, leading to the termination of this series (data not shown). In contrast, several analogs containing the *N*-(benzo[*d*]thiazol-2-yl)-5-phenyl-1,3,4-oxadiazol-2-amine structure of MLS-0049585 with similar or even better potency could be identified (Table III in the online-only Data Supplement). The 4 most potent compounds were selected for testing in human platelets. Inhibition of platelet aggregation was assessed with platelets collected from 3 healthy donors. In these experiments, MLS-0437605 (Figure 7A) efficiently inhibited platelet aggregation in response to CRP and rhodocytin, but not after stimulation with thromboxane (Figure 7B and 7C). Tests on platelets from WT mice yielded similar results (Figure 7D). In contrast, MLS-0437605 only minimally affected the aggregation of DUSP3-deficient platelets (Figure 7D). Selectivity was further evaluated against 10 additional PTPs (Table). In these assays, MLS-0437605 showed excellent selectivity for DUSP3 over the vast majority of PTPs tested. Importantly, there was good selectivity of MLS-0437605 for DUSP3 over DUSP22 (7-fold), another dual-specificity phosphatase that is highly expressed in platelets (Figure 1A). We next examined the effect of MLS-0437605 on GPVI- and CLEC-2–induced signaling in human platelets. Global tyrosine phosphorylation was analyzed on total lysates from resting or activated platelets. MLS-0437605 caused a decrease in phosphotyrosine of an ≈70-kDa band after stimulation with CRP or rhodocytin (Figure 7E and 7F). Tyrosine phosphorylation of immunoprecipitated Syk and PLCγ2 was also reduced by MLS-0437605 (Figure 7G and 7H). These data demonstrate that pharmacological inhibition of DUSP3 activity in human platelets affects platelet signaling in a manner similar to DUSP3 deficiency in *Dusp3*-KO platelets.

Table. Selectivity of the DUSP3 Inhibitor MLS-0437605

	IC_{50}, μmol/L
DUSP3	3.7
PTP-SL	13
DUSP22	26
HePTP	38
LYP	49
TCPTP	55
CD45	>100
LAR	>100
STEP	>100
PTP1B	>100
DUSP6	>100

Discussion

This is the first study implicating a member of the PTP subfamily of dual-specificity phosphatases in GPVI- and CLEC-2–induced signaling. Motivated by our finding that DUSP3 is highly expressed in human and mouse platelets, we used *Dusp3*-KO mice to study the role of this phosphatase in hemostasis and thrombosis. DUSP3-deficient mice were more resistant to pulmonary thromboembolism than their WT littermates. Thrombus formation was strongly impaired in the model of $FeCl_3$-induced injury of carotid artery in a platelet-specific manner. Intriguingly, DUSP3-deficient mice did not bleed spontaneously and showed normal tail bleeding times. These findings suggest that DUSP3 plays a key role in arterial thrombosis but is dispensable for primary hemostasis.

Ex vivo, on platelet stimulation with a low concentration of collagen, convulxin, CRP, or rhodocytin, DUSP3 deficiency resulted in defective platelet aggregation, granule secretion, and integrin $\alpha_{IIb}\beta_3$ inside-out activation. In contrast, platelet activation mediated by G-protein–coupled receptor agonists was not affected. DUSP3 deficiency led to a reduction in thrombus formation on collagen-coated surfaces under arterial shear, as well as lower phosphatidylserine exposure at the surface of adhered platelets. These data indicate that GPVI- and CLEC-2–mediated platelet activation is impaired in DUSP3-deficient platelets.[25–27] DUSP3 was dispensable for integrin $\alpha_{IIb}\beta_3$ outside-in signaling, as indicated by unaltered fibrin clot retraction (data not shown). *Dusp3*-KO mice exhibited levels of thrombus formation comparable to the previously reported GPVI-KO/FcRγ-KO,[28–30] CLEC-2–KO,[31] CLEC-2–depleted,[26] GPVI-depleted,[32] and CLEC-2/GPVI–depleted mice.[27] Similar to our findings in DUSP3-deficient mice, GPVI-KO and CLEC-2–KO mice do not exhibit prolonged bleeding time.[28,31,33] DUSP3-deficient mice were also protected against pulmonary thromboembolism

induced by a mixture of collagen and epinephrine, similar to GPVI-KO mice.[33]

At the molecular level, phosphorylation of the previously reported DUSP3 substrates ERK1/2 and JNK1/2[7] was not affected by DUSP3 deficiency, suggesting that signaling defects in DUSP3-deficient platelets are independent of the ERK1/2 and JNK1/2 pathways. However, we cannot exclude the possibility of functional or compensatory redundancies between DUSP3 and other phosphatases.

GPVI and CLEC-2 signaling pathways share many similarities, including the activation of Syk, phospholipase Cγ2, and adapter proteins such as LAT and SLP-76.[34] However, there is also a significant difference: in GPVI-stimulated platelets, SFKs initiate signaling through phosphorylation of the FcRγ-associated immunoreceptor tyrosine-based activation motifs, leading to binding and activation of Syk.[20,21] In contrast, signaling through CLEC-2 depends on phosphorylation of CLEC-2 by Syk in an SFK-independent manner.[35] Because DUSP3 deficiency limits platelet activation in response to both GPVI and CLEC-2 stimulation, SFK function is likely not controlled by DUSP3, which is also supported by our data showing that phosphotyrosine in SFKs is not altered in *Dusp3*-KO platelets. On the contrary, Syk may be directly or indirectly targeted by DUSP3. Intriguingly, however, DUSP3 deficiency decreased phosphotyrosine levels in Syk. Furthermore, no hyperphosphorylated protein could be identified in phosphotyrosine blots of total lysates from DUSP3-deficient platelets. This raises the question of whether phosphoserine or phosphothreonine in Syk or other protein(s) may be targeted by DUSP3, a dual-specificity phosphatase able to dephosphorylate both phosphotyrosine and phosphoserine/phosphothreonine. Given the limited recognition sites of available phosphoserine/phosphothreonine antibodies, future studies using quantitative phosphoproteomics analysis are necessary to address this question.

Platelet binding to von Willebrand factor via GPIbα allows engagement of the collagen receptors GPVI and $\alpha_2\beta_1$, leading to platelet arrest and subsequent platelet thrombus formation. The von Willebrand factor–GPIb axis also induces GPVI dimerization, resulting in direct enhancement of GPVI interaction with collagen.[36] However, platelets from *Dusp3*-KO mice exhibit normal binding to von Willebrand factor–coated surface under flow (Figure VII in the online-only Data Supplement), suggesting intact GPIb signaling in these animals. Interestingly, a recent study by Nieswandt's group showed that combined depletion of GPVI and CLEC-2 was sufficient to abrogate arterial thrombosis in mice.[27] Thus, the defects observed in DUSP3-deficient platelets on CLEC-2– and GPVI-induced signaling are sufficient to explain the impaired thrombus formation in *Dusp3*-KO mice.

Finally, platelets are anucleate cells that are not amenable to RNA interference or recombinant DNA technologies. Thus, to corroborate our findings in human cells, we used a chemical genomics approach. Specifically, a small-molecule inhibitor of DUSP3 was identified via high-throughput screening of a large chemical library and subsequent structure-activity relationship studies. Previously reported DUSP3 inhibitors suffer from poor selectivity, lack of efficacy, or both,[37–41] or they cause immediate spontaneous aggregation of platelets (data not shown).[42] Thus, we developed a novel, specific, and efficacious inhibitor that we used to inhibit DUSP3 function in human washed platelets. Similar to DUSP3 deficiency in murine cells, inhibition of DUSP3 activity in human platelets led to suppression of platelet aggregation, specifically in response to CRP and rhodocytin, but not in response to the G-protein–coupled receptor agonist thromboxane. MLS-0437605 is a drug-like compound[43] and may serve as the basis for the development of potential therapeutics targeting DUSP3 for the treatment of arterial thrombosis.

Conclusions

We demonstrated that DUSP3 is a key signaling molecule for GPVI- and CLEC-2–induced platelet activation. We developed a specific small-molecule inhibitor of DUSP3 that efficiently inhibited human platelet activation in vitro. Given that *Dusp3*-KO mice remain healthy, do not exhibit any spontaneous phenotype, and do not suffer from increased bleeding events, our findings may lead to a novel antiplatelet therapy.

Acknowledgments

We thank the GIGA-Animal, GIGA-Genotranscriptomics, and GIGA-Imaging core facilities for their assistance and technical help.

Sources of Funding

This work was supported by the Belgian National Fund for Scientific Research (F.R.S.-FNRS: PDR No. T.0105.13), the University of Liège (Fonds Spéciaux pour la Recherche to Drs Oury and Rahmouni), the Deutsche Forschungsgemeinschaft (grant SFB1009 A09 to Dr Eble), the Cardiovascular Center of the Maastricht University Medical Center (to Dr Heemskerk), the American Heart Association (Innovative Research Grant 14IRG18980075 to Dr Tautz), and grants from the National Institutes of Health (5R01AI035603 to Dr Mustelin, U54 HG005033 to J.C. Reed/Conrad Prebys Center for Chemical Genomics, and 1R21CA132121 and 1R03MH084230 to Dr Tautz).

Disclosures

None.

References

1. Michelson AD. Antiplatelet therapies for the treatment of cardiovascular disease. *Nat Rev Drug Discov*. 2010;9:154–169. doi: 10.1038/nrd2957.
2. Watson SP, Auger JM, McCarty OJ, Pearce AC. GPVI and integrin alphaIIb beta3 signaling in platelets. *J Thromb Haemost*. 2005;3:1752–1762. doi: 10.1111/j.1538-7836.2005.01429.x.
3. Karisch R, Fernandez M, Taylor P, Virtanen C, St-Germain JR, Jin LL, Harris IS, Mori J, Mak TW, Senis YA, Östman A, Moran MF, Neel BG. Global proteomic assessment of the classical protein-tyrosine phosphatome and "Redoxome." *Cell*. 2011;146:826–840. doi: 10.1016/j.cell.2011.07.020.
4. Alonso A, Sasin J, Bottini N, Friedberg I, Friedberg I, Osterman A, Godzik A, Hunter T, Dixon J, Mustelin T. Protein tyrosine phosphatases in the human genome. *Cell*. 2004;117:699–711. doi: 10.1016/j.cell.2004.05.018.
5. Tautz L, Critton DA, Grotegut S. Protein tyrosine phosphatases: structure, function, and implication in human disease. *Methods Mol Biol*. 2013;1053:179–221. doi: 10.1007/978-1-62703-562-0_13.
6. Ishibashi T, Bottaro DP, Chan A, Miki T, Aaronson SA. Expression cloning of a human dual-specificity phosphatase. *Proc Natl Acad Sci USA*. 1992;89:12170–12174.
7. Cerignoli F, Rahmouni S, Ronai Z, Mustelin T. Regulation of MAP kinases by the VHR dual-specific phosphatase: implications for cell growth and differentiation. *Cell Cycle*. 2006;5:2210–2215.

8. Wang JY, Yeh CL, Chou HC, Yang CH, Fu YN, Chen YT, Cheng HW, Huang CY, Liu HP, Huang SF, Chen YR. Vaccinia H1-related phosphatase is a phosphatase of ErbB receptors and is down-regulated in non-small cell lung cancer. *J Biol Chem*. 2011;286:10177–10184. doi: 10.1074/jbc.M110.163295.
9. Rahmouni S, Cerignoli F, Alonso A, Tsutji T, Henkens R, Zhu C, Louis-dit-Sully C, Moutschen M, Jiang W, Mustelin T. Loss of the VHR dual-specific phosphatase causes cell-cycle arrest and senescence. *Nat Cell Biol*. 2006;8:524–531. doi: 10.1038/ncb1398.
10. Henkens R, Delvenne P, Arafa M, Moutschen M, Zeddou M, Tautz L, Boniver J, Mustelin T, Rahmouni S. Cervix carcinoma is associated with an up-regulation and nuclear localization of the dual-specificity protein phosphatase VHR. *BMC Cancer*. 2008;8:147. doi: 10.1186/1471-2407-8-147.
11. Arnoldussen YJ, Lorenzo PI, Pretorius ME, Waehre H, Risberg B, Maelandsmo GM, Danielsen HE, Saatcioglu F. The mitogen-activated protein kinase phosphatase vaccinia H1-related protein inhibits apoptosis in prostate cancer cells and is overexpressed in prostate cancer. *Cancer Res*. 2008;68:9255–9264. doi: 10.1158/0008-5472.CAN-08-1224.
12. Amand M, Erpicum C, Bajou K, Cerignoli F, Blacher S, Martin M, Dequiedt F, Drion P, Singh P, Zurashvili T, Vandereyken M, Musumeci L, Mustelin T, Moutschen M, Gilles C, Noel A, Rahmouni S. DUSP3/VHR is a pro-angiogenic atypical dual-specificity phosphatase. *Mol Cancer*. 2014;13:108. doi: 10.1186/1476-4598-13-108.
13. Oury C, Toth-Zsamboki E, Vermylen J, Hoylaerts MF. P2X(1)-mediated activation of extracellular signal-regulated kinase 2 contributes to platelet secretion and aggregation induced by collagen. *Blood*. 2002;100:2499–2505. doi: 10.1182/blood-2002-03-0812.
14. Oury C, Kuijpers MJ, Toth-Zsamboki E, Bonnefoy A, Danloy S, Vreys I, Feijge MA, De Vos R, Vermylen J, Heemskerk JW, Hoylaerts MF. Overexpression of the platelet P2X1 ion channel in transgenic mice generates a novel prothrombotic phenotype. *Blood*. 2003;101:3969–3976. doi: 10.1182/blood-2002-10-3215.
15. Gilio K, van Kruchten R, Braun A, Berna-Erro A, Feijge MA, Stegner D, van der Meijden PE, Kuijpers MJ, Varga-Szabo D, Heemskerk JW, Nieswandt B. Roles of platelet STIM1 and Orai1 in glycoprotein VI- and thrombin-dependent procoagulant activity and thrombus formation. *J Biol Chem*. 2010;285:23629–23638. doi: 10.1074/jbc.M110.108696.
16. Munnix IC, Kuijpers MJ, Auger J, Thomassen CM, Panizzi P, van Zandvoort MA, Rosing J, Bock PE, Watson SP, Heemskerk JW. Segregation of platelet aggregatory and procoagulant microdomains in thrombus formation: regulation by transient integrin activation. *Arterioscler Thromb Vasc Biol*. 2007;27:2484–2490. doi: 10.1161/ATVBAHA.107.151100.
17. Heemskerk JW, Sage SO. Calcium signalling in platelets and other cells. *Platelets*. 1994;5:295–316. doi: 10.3109/09537109409006439.
18. Kuijpers MJ, Munnix IC, Cosemans JM, Vlijmen BV, Reutelingsperger CP, Egbrink MO, Heemskerk JW. Key role of platelet procoagulant activity in tissue factor-and collagen-dependent thrombus formation in arterioles and venules in vivo differential sensitivity to thrombin inhibition. *Microcirculation*. 2008;15:269–282. doi: 10.1080/10739680701653517.
19. Senis YA, Tomlinson MG, Ellison S, Mazharian A, Lim J, Zhao Y, Kornerup KN, Auger JM, Thomas SG, Dhanjal T, Kalia N, Zhu JW, Weiss A, Watson SP. The tyrosine phosphatase CD148 is an essential positive regulator of platelet activation and thrombosis. *Blood*. 2009;113:4942–4954. doi: 10.1182/blood-2008-08-174318.
20. Watson SP, Asazuma N, Atkinson B, Berlanga O, Best D, Bobe R, Jarvis G, Marshall S, Snell D, Stafford M, Tulasne D, Wilde J, Wonerow P, Frampton J. The role of ITAM- and ITIM-coupled receptors in platelet activation by collagen. *Thromb Haemost*. 2001;86:276–288.
21. Ezumi Y, Shindoh K, Tsuji M, Takayama H. Physical and functional association of the Src family kinases Fyn and Lyn with the collagen receptor glycoprotein VI-Fc receptor gamma chain complex on human platelets. *J Exp Med*. 1998;188:267–276.
22. Nieswandt B, Brakebusch C, Bergmeier W, Schulte V, Bouvard D, Mokhtari-Nejad R, Lindhout T, Heemskerk JW, Zirngibl H, Fässler R. Glycoprotein VI but not alpha2beta1 integrin is essential for platelet interaction with collagen. *EMBO J*. 2001;20:2120–2130. doi: 10.1093/emboj/20.9.2120.
23. Schoenwaelder SM, Yuan Y, Josefsson EC, White MJ, Yao Y, Mason KD, O'Reilly LA, Henley KJ, Ono A, Hsiao S, Willcox A, Roberts AW, Huang DC, Salem HH, Kile BT, Jackson SP. Two distinct pathways regulate platelet phosphatidylserine exposure and procoagulant function. *Blood*. 2009;114:663–666. doi: 10.1182/blood-2009-01-200345.
24. Bobkova EV, Liu WH, Colayco S, Rascon J, Vasile S, Gasior C, Critton DA, Chan X, Dahl R, Su Y, Sergienko E, Chung TD, Mustelin T, Page R, Tautz L. Inhibition of the hematopoietic protein tyrosine phosphatase by phenoxyacetic acids. *ACS Med Chem Lett*. 2011;2:113–118. doi: 10.1021/ml100103p.
25. Siljander P, Farndale RW, Feijge MA, Comfurius P, Kos S, Bevers EM, Heemskerk JW. Platelet adhesion enhances the glycoprotein VI-dependent procoagulant response: involvement of p38 MAP kinase and calpain. *Arterioscler Thromb Vasc Biol*. 2001;21:618–627.
26. May F, Hagedorn I, Pleines I, Bender M, Vögtle T, Eble J, Elvers M, Nieswandt B. CLEC-2 is an essential platelet-activating receptor in hemostasis and thrombosis. *Blood*. 2009;114:3464–3472. doi: 10.1182/blood-2009-05-222273.
27. Bender M, May F, Lorenz V, Thielmann I, Hagedorn I, Finney BA, Vögtle T, Remer K, Braun A, Bösl M, Watson SP, Nieswandt B. Combined in vivo depletion of glycoprotein VI and C-type lectin-like receptor 2 severely compromises hemostasis and abrogates arterial thrombosis in mice. *Arterioscler Thromb Vasc Biol*. 2013;33:926–934. doi: 10.1161/ATVBAHA.112.300672.
28. Kato K, Kanaji T, Russell S, Kunicki TJ, Furihata K, Kanaji S, Marchese P, Reininger A, Ruggeri ZM, Ware J. The contribution of glycoprotein VI to stable platelet adhesion and thrombus formation illustrated by targeted gene deletion. *Blood*. 2003;102:1701–1707. doi: 10.1182/blood-2003-03-0717.
29. Dubois C, Panicot-Dubois L, Merrill-Skoloff G, Furie B, Furie BC. Glycoprotein VI-dependent and -independent pathways of thrombus formation in vivo. *Blood*. 2006;107:3902–3906. doi: 10.1182/blood-2005-09-3687.
30. Konishi H, Katoh Y, Takaya N, Kashiwakura Y, Itoh S, Ra C, Daida H. Platelets activated by collagen through immunoreceptor tyrosine-based activation motif play pivotal role in initiation and generation of neointimal hyperplasia after vascular injury. *Circulation*. 2002;105:912–916.
31. Suzuki-Inoue K, Inoue O, Ding G, Nishimura S, Hokamura K, Eto K, Kashiwagi H, Tomiyama Y, Yatomi Y, Umemura K, Shin Y, Hirashima M, Ozaki Y. Essential *in vivo* roles of the C-type lectin receptor CLEC-2: embryonic/neonatal lethality of CLEC-2-deficient mice by blood/lymphatic misconnections and impaired thrombus formation of CLEC-2-deficient platelets. *J Biol Chem*. 2010;285:24494–24507. doi: 10.1074/jbc.M110.130575.
32. Massberg S, Gawaz M, Grüner S, Schulte V, Konrad I, Zohlnhöfer D, Heinzmann U, Nieswandt B. A crucial role of glycoprotein VI for platelet recruitment to the injured arterial wall in vivo. *J Exp Med*. 2003;197:41–49.
33. Lockyer S, Okuyama K, Begum S, Le S, Sun B, Watanabe T, Matsumoto Y, Yoshitake M, Kambayashi J, Tandon NN. GPVI-deficient mice lack collagen responses and are protected against experimentally induced pulmonary thromboembolism. *Thromb Res*. 2006;118:371–380. doi: 10.1016/j.thromres.2005.08.001.
34. Navarro-Núñez L, Langan SA, Nash GB, Watson SP. The physiological and pathophysiological roles of platelet CLEC-2. *Thromb Haemost*. 2013;109:991–998. doi: 10.1160/TH13-01-0060.
35. Séverin S, Pollitt AY, Navarro-Nuñez L, Nash CA, Mourão-Sá D, Eble JA, Senis YA, Watson SP. Syk-dependent phosphorylation of CLEC-2: a novel mechanism of hem-immunoreceptor tyrosine-based activation motif signaling. *J Biol Chem*. 2011;286:4107–4116. doi: 10.1074/jbc.M110.167502.
36. Loyau S, Dumont B, Ollivier V, Boulaftali Y, Feldman L, Ajzenberg N, Jandrot-Perrus M. Platelet glycoprotein VI dimerization, an active process inducing receptor competence, is an indicator of platelet reactivity. *Arterioscler Thromb Vasc Biol*. 2012;32:778–785. doi: 10.1161/ATVBAHA.111.241067.
37. Usui T, Kojima S, Kidokoro S, Ueda K, Osada H, Sodeoka M. Design and synthesis of a dimeric derivative of RK-682 with increased inhibitory activity against VHR, a dual-specificity ERK phosphatase: implications for the molecular mechanism of the inhibition. *Chem Biol*. 2001;8:1209–1220.
38. Ueda K, Usui T, Nakayama H, Ueki M, Takio K, Ubukata M, Osada H. 4-Isoavenaciolide covalently binds and inhibits VHR, a dual-specificity phosphatase. *FEBS Lett*. 2002;525:48–52.
39. Hirai G, Tsuchiya A, Koyama Y, Otani Y, Oonuma K, Dodo K, Simizu S, Osada H, Sodeoka M. Development of a Vaccinia H1-related (VHR) phosphatase inhibitor with a nonacidic phosphate-mimicking core structure. *ChemMedChem*. 2011;6:617–622. doi: 10.1002/cmdc.201100107.
40. Park H, Jung SK, Jeong DG, Ryu SE, Kim SJ. Discovery of VHR phosphatase inhibitors with micromolar activity based on structure-based virtual screening. *ChemMedChem*. 2008;3:877–880. doi: 10.1002/cmdc.200700348.
41. Shi Z, Tabassum S, Jiang W, Zhang J, Mathur S, Wu J, Shi Y. Identification of a potent inhibitor of human dual-specific phosphatase, VHR,

from computer-aided and NMR-based screening to cellular effects. *Chembiochem*. 2007;8:2092–2099. doi: 10.1002/cbic.200700397.
42. Wu S, Vossius S, Rahmouni S, Miletic AV, Vang T, Vazquez-Rodriguez J, Cerignoli F, Arimura Y, Williams S, Hayes T, Moutschen M, Vasile S, Pellecchia M, Mustelin T, Tautz L. Multidentate small-molecule inhibitors of vaccinia H1-related (VHR) phosphatase decrease proliferation of cervix cancer cells. *J Med Chem*. 2009;52:6716–6723. doi: 10.1021/jm901016k.
43. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliv Rev*. 2001;46:3–26.

CLINICAL PERSPECTIVE

A limitation of current antiplatelet therapies is their inability to separate thrombotic events from bleeding occurrences. The present study demonstrates that dual-specificity phosphatase 3 (DUSP3) phosphatase deficiency in mice does not cause bleeding but still protects against arterial thrombosis and collagen-induced thromboembolism. This protection is at least partially attributable to the selective inhibition of glycoprotein VI– and C-type lectin-like receptor 2–dependent signaling. Furthermore, ex vivo, a selective inhibitor of DUSP3 limits glycoprotein VI– and C-type lectin-like receptor 2–mediated aggregation of human platelets. Our findings pave the way for further preclinical studies in animal models and future validation in humans toward the development of a novel, DUSP3-based therapeutic strategy in arterial thrombosis.